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Cariogenic Effects of Probiotic *Lactobacillus rhamnosus GG* in a Dental Biofilm Model

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Key Words

Bacteria · Biofilm(s) · Caries · Microbial ecology · Plaque/plaque biofilms · Probiotics

Abstract

Probiotic bacteria have been suggested to inhibit *Streptococcus mutans* (SM) and thus prevent dental caries. However, supporting evidence is weak and probiotic species might be cariogenic themselves. Thus, we compared and combined the probiotic *Lactobacillus rhamnosus* GG (LGG) with SM and analysed the resulting mineral loss (ΔZ) in dental tissues. We simulated three biofilm compositions (SM, LGG, SM × LGG), two lesion sites (smooth enamel, dentin cavity) and two nutrition supply frequencies (twice/day, 6 times/day) in a multi-station, continuous-culture biofilm model. A total of 240 bovine enamel and dentin samples were cut, polished and embedded. All experimental procedures were performed in independent duplicates, with 10 samples being allocated to each group for each experiment (final sample size n = 20/group). Biofilms were cultured on the specimens and supplied with 2% sucrose medium and artificial saliva in consecutive pulses. After 10 days, ΔZ and bacterial numbers were assessed. SM × LGG biofilms caused significantly increased ΔZ compared with SM or LGG biofilms ($p < 0.01$, Mann-Whitney test), and ΔZ was significantly increased in dentin cavities compared with smooth enamel lesions ($p < 0.01$). Bacterial numbers did not significantly differ between biofilms of different species ($p > 0.05$, ANOVA). Frequent nutrition supply significantly increased bacterial numbers ($p < 0.01$). Biofilms in dentin cavities compared to smooth enamel harboured significantly more bacteria ($p < 0.05$). LGG induced mineral loss especially in dentin cavities and under highly cariogenic conditions. LGG did not have inhibitory effects on SM, but rather contributed to the caries process in vitro.

Probiotics are 'live microorganisms which, when administered in adequate amounts, confer a health benefit on the host' [Schlundt, 2001], and are thought to either restore the natural microbiome or actively interfere with pathogenic organisms. Such interference includes co-aggregation, toxicity via by-products, sequestration of substrate or metal ions, or modulatory effects on the host's immune system [Meurman, 2005; Dierksen et al., 2007; Twetman and Keller, 2012].

Cariogenic bacteria like *Streptococcus mutans* (SM) are only weakly competitive at neutral pH, but can successfully compete under reduced pH caused by catabolism of dietary carbohydrates [Takahashi and Nyvad,

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2011]. Probiotics have been suggested to positively alter and re-shift the ecologic conditions within the dental biofilm and thereby prevent or reduce caries development, but the evidence for the inhibition of SM and interference with caries development remains ambiguous [Michalek et al., 1981; Näse et al., 2001; Simark-Mattsson et al., 2007; Stecksén-Blicks et al., 2009; Marttinen et al., 2012]. Evidence in support of the efficacy of probiotics to prevent caries is further limited due to the short-term character of most studies and the limited validity of analysed parameters (e.g. bacterial numbers instead of caries induction) for the caries process [Twetman and Keller, 2012]. Additionally, there are concerns that probiotics like lactobacilli might not have beneficial but detrimental effects on caries development, and concentrations of oral lactobacilli, for example, have been correlated with the number of white spot and dentinal caries lesions as well as caries activity [Bönecker et al., 2003; Beighton et al., 2004; Kõll-Klais et al., 2005; Badet and Thebaud, 2008].

One of the most frequently investigated probiotic organisms is *Lactobacillus rhamnosus* GG (LGG), which was shown to reduce both the number and acidogenic activity of SM [Meurman et al., 1995; Näse et al., 2001; Kõll-Klais et al., 2005]. Possible cariogenic effects of LGG remain controversial, and it is unclear how factors like pre-existing carious lesions or a cariogenic diet might influence these effects [Meurman et al., 1995; Haukioja et al., 2008; Hedberg et al., 2008; Kneist et al., 2010]. Therefore, the present study investigated the cariogenicity of LGG in comparison and combination with SM in a continuous-culture biofilm model. We hypothesized that there is no significant difference in cariogenicity or bacterial numbers of LGG, SM and mixed SM × LGG biofilms. We further hypothesized that neither different nutrition supply frequency nor different lesion sites will significantly influence cariogenicity or bacterial numbers.

Material and Methods

Study Design

In a randomized design, mono- and multi-species biofilms were grown within a continuous-culture biofilm model. The influence of different factors on integrated mineral loss (ΔZ) of developed caries lesions was to be analysed. Evaluated factors were bacterial species (SM, LGG, LGG × SM), lesion site (smooth enamel or dentin cavity) and availability of fermentable carbohydrates (nutrition supply twice or 6 times daily). Therefore, 12 experimental subsets were investigated. All experimental procedures were performed in independent biological duplicates. For each of these experiments, 10 samples per group were used. Thus, a total of 240 specimens (n = 20 per group) was used for the study. All of these samples were prepared to analyse ΔZ. A total of 9 samples per group was used to analyse bacterial numbers and pH of the biofilms (fig. 1).

Bacterial Culture

SM ATCC 25175 and LGG ATCC 53103 were cultured for 48 h (37 °C) on brain-heart infusion (BHI, Difco, Franklin Lakes, N.J., USA) and de-Man-Rogosa-Sharpe agar (MRS, Difco), respectively. Cultures were then inoculated into 10 ml BHI medium and grown similarly for 24 h.

Specimen Preparation

From 60 bovine incisors of the second dentition, 120 enamel-dentin specimens (2 × 3 × 4 mm) and 120 dentin specimens (2 × 3 × 2 mm) were prepared (Band Saw Exakt 300 cl; Exakt Apparatebau, Norderstedt, Germany), ground flat (Phoenix Alpha; Buehler, Düsseldorf, Germany), and polished (abrasive paper 1,200, 2,400, 4,000 Exakt Apparatebau). Half of each specimen was covered with nail varnish (Rival de Loop, Rossmann, Burgwedel, Germany) to serve as sound reference after demineralization. Groups of 10 enamel or dentin specimens were embedded in acrylic resin carrier bars (Technovit 4071, Heraeus Kulzer, Hanau, Germany), with the enamel surfaces being levelled, whilst dentin surfaces were embedded in cavities (1 × 2 × 3 mm) using standardized silicon placeholders. The resulting 24 carrier bars were sterilized (121 °C, 2.1 bar, 34 min). For each of the duplicative experiments, 12 bars were used, with one bar being allocated to the 12 experimental subsets. In total, 240 samples were used (n = 2 × 10 per group, fig. 1).

Artificial Mouth Setting

A computer-controlled, multi-station continuous-culture biofilm model [Dibdin et al., 1976; Sissons et al., 1991] with three different chambers and nutrition and saliva supply via peristaltic multi-channel pumps (STA, Desaga-Sarstedt, Nümbrecht, Germany) was used to simulate oral conditions. Two carrier bars, one with enamel and one with dentin samples, were placed within one chamber at 100% humidity and 37 °C. Each carrier was conditioned with 5 ml sterile-filtrated natural saliva obtained with informed consent under a reviewed and ethics-approved protocol for 2 h to form a pellicle. Saliva was removed, bars inoculated with 5 ml of allocated bacterial suspension and biofilms grown for 24 h.

Groups were provided with pulses of sterile BHI + 2% sucrose (1.25 ml/min for 15 min) twice or 6 times daily, respectively, with an overnight resting period of at least 7 h 50 min. All groups were provided with sterile modified defined mucin medium [Wong and Sissons, 2001] 6 times daily to simulate artificial salivary (online suppl. table 1, www.karger.com/doi/10.1159/000355907). Since simulated meals and salivary flow were coordinated, sugar was available for a total of 25 min (15 min supply, 10 min delay time) and then slowly rinsed away by salivary flow. Mixed LGG × SM cultures were re-inoculated daily with 5-ml overnight cultures of LGG (approximately 7×10^6 CFU/ml) to simulate the uptake of probiotic (fig. 2). After 10 days, the simulation period was concluded and specimens and biofilms were analysed.

Biofilm Analysis

Random biofilms were isolated from defined surface areas (2 × 2 mm), with 3 biofilms removed in the first experiment and 6 biofilms removed in the second, duplicative experiment. Thus, a total of 9 biofilms per group was analysed.

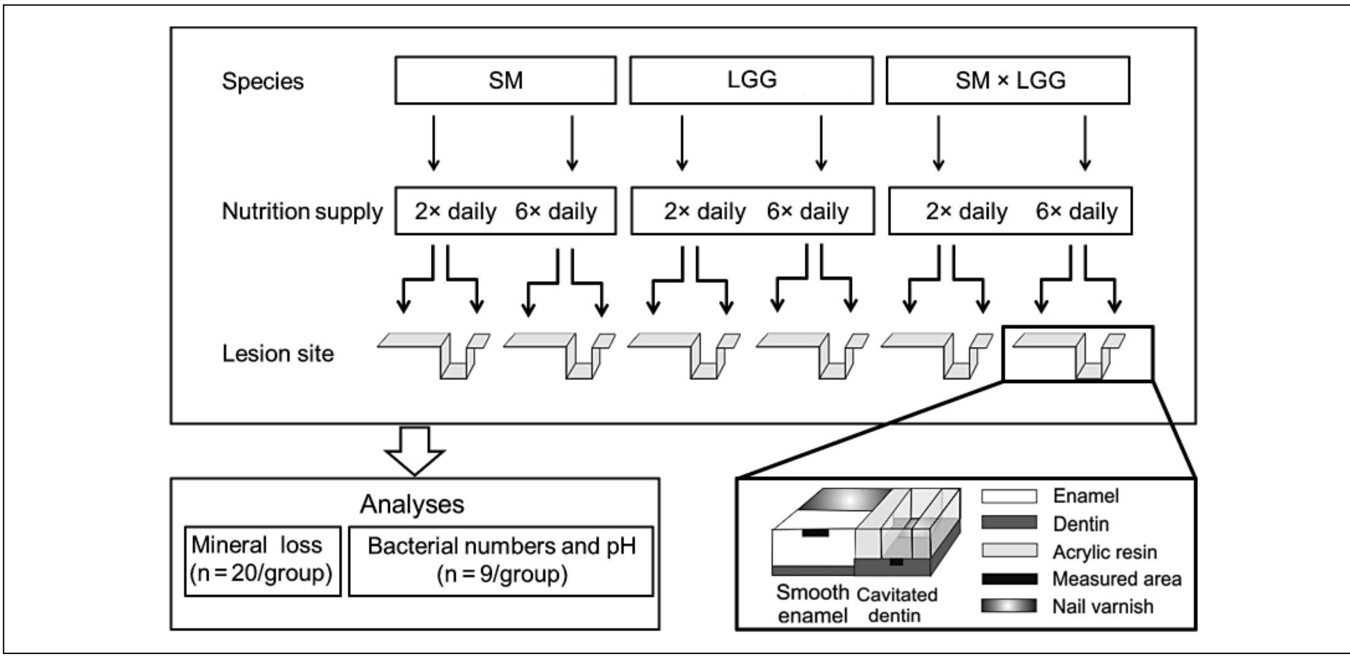
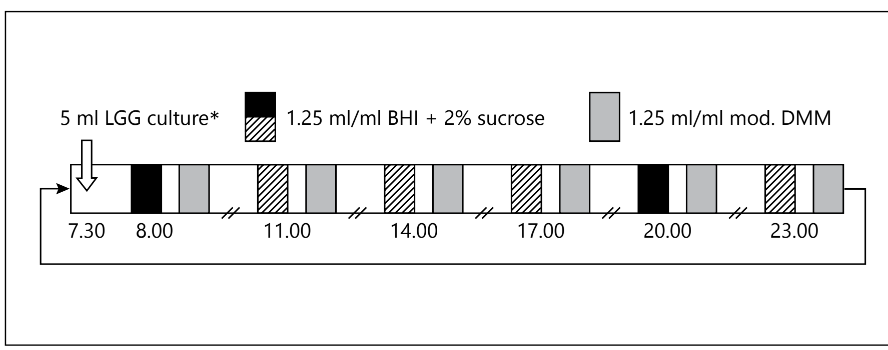


Fig. 1. Study design. Twelve different subsets were simulated in a continuous-culture biofilm model. After 10 days, ΔZ as well as bacterial numbers and pH of biofilms were analysed. Specimens (window) consisted of smooth enamel and dentin cavities, with half of each surface being covered with nail varnish to serve as sound reference. Two duplicative experiments were performed, with 10 samples per group being analysed in each experiment. Thus, a total of 240 samples were used: 20 samples per group were assessed to analyse ΔZ, and 9 biofilms were analysed regarding bacterial numbers and pH.

Fig. 2. Sequence of media provision during continuous culturing from day 2 to 10. BHI-sucrose medium (black) and artificial saliva (grey) were supplied in different frequencies and intervals (hatched: additional four pulses of sucrose-medium resulted in nutrition supply frequency of 6 times daily). Each pulse lasted 15 min, with a break of 10 min after BHI-sucrose supply. A fasting period was simulated overnight. Daily supply of LGG in mixed SM × LGG cultures only. Mod. DMM = Modified defined mucin medium.



Biofilms were inoculated in 1 ml BHI, vortex mixed and pH measured (InLab Micro, Mettler-Toledo, Giessen, Germany). Bacteria from mixed biofilms were plated and cultured on mitis-salivarius agar (Difco) with bacitracin (MSB) [Gold et al., 1973], MRS and BHI agar plates in various dilutions (10^5–10^7) at 37°C and 5% CO_2. Bacteria from monospecies biofilms were similarly cultured on BHI agar only. After 48 h, colony-forming units per milliliter were enumerated.

Transverse Microradiography

Discs were cut perpendicularly into thin sections (100 µm; Band Saw 300 cl; Mikroschleifsystem 400 CS, abrasive paper 1,200, 2,400 and 4,000). A nickel-filtered copper X-ray source (PW3830, Pananalytical, Kassel, Germany) operating at 20 kV and 20 mA was used to obtain radiographs. Films (35 mm B/W positive, Fujifilm, Tokyo, Japan) were exposed for 10 s and developed under standardized conditions according to the manufacturer's recommendations. Microradiographs were analysed using a digital-image-analysing system (CFW 1312M, Scion, Frederick, USA) interfaced with a universal microscope (Axioplan 60318, Zeiss, Oberkochen, Germany) and a personal computer (transverse microradiography, TMR for Windows 5.25; UMCG, Groningen, The Netherlands). During the preparation process, 55 of the 240 samples were lost and could not be analysed. The number of analysed samples of each group is given within the results.

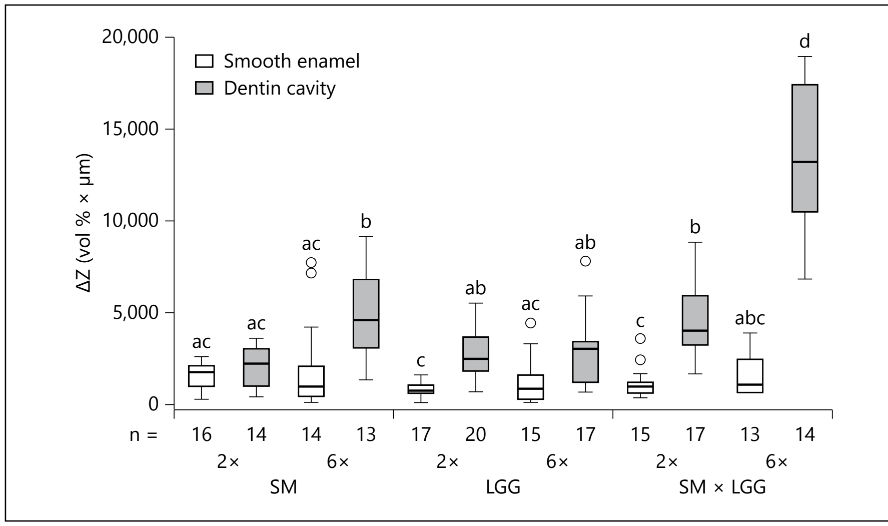


Fig. 3. ΔZ of smooth enamel (white) and cavitated dentin (grey) after 10 days of biofilm cultivation of SM, LGG and mixed SM × LGG with twice or 6 times nutrition supply per day. Significant differences between groups are indicated by different letters ($p < 0.05$, Mann-Whitney/Bonferroni). The number of analysed specimens (n) is given, since some specimens were lost and/or not measurable. Box and thick line = 25th/75th percentiles and median; error bars = minima and maxima; circles = outliers.

Statistical Analysis

Statistical analysis was performed with SPSS 20 (IBM, Armonk, N.Y., USA). Normal distribution was controlled using Shapiro-Wilk test. Results of both experimental subsets were analysed separately and not found to statistically differ significantly ($p > 0.05$, ANOVA and Mann-Whitney U). Comparison of experimental subsets to analyse the effects of bacterial species, lesion site and nutrition on ΔZ were performed using Kruskal-Wallis and Mann-Whitney U test. Respective effects on colony-forming units per milliliter and pH were assessed using univariate ANOVAs. Level of significance was set at $p < 0.05$, with Bonferroni correction for multiple testing.

Results

Mixed SM × LGG biofilms caused significantly increased ΔZ compared with both monospecies biofilms in dentin cavities ($p < 0.01$, Mann-Whitney/Bonferroni), whilst there was no significant difference for smooth enamel lesions ($p > 0.05$). SM and LGG did not induce significantly different ΔZ in neither smooth enamel nor dentin cavities ($p > 0.05$). The frequency of nutrition supply did not significantly influence ΔZ of smooth enamel lesions ($p > 0.05$), whilst ΔZ of dentin cavity lesions was significantly increased, if nutrition was supplied 6 times compared with twice daily ($p < 0.01$). Dentin cavity lesions showed significantly increased ΔZ compared with smooth enamel lesions irrespective of the bacterial species or nutrition frequency ($p < 0.01$). The highest ΔZ was induced by SM × LGG biofilms with 6 times nutrition supply in dentin cavities (fig. 3).

Bacterial numbers did not significantly differ in SM, LGG or SM × LGG biofilms (table 1). Nutrition supply had a significant influence on bacterial numbers, with biofilm supplied 6 times daily showing increased bacterial numbers ($8.7 \pm 1.6 \times 10^6$ CFU/ml; mean ± SD) compared with twice daily ($2.6 \pm 1.5 \times 10^6$ CFU/ml). Lesion site had a significant influence on bacterial numbers, with dentin cavities harbouring more bacteria ($8.5 \pm 1.5 \times 10^6$ CFU/ml) than biofilms on smooth enamel ($3.2 \pm 1.6 \times 10^6$ CFU/ml).

There was a trend of decreased pH in biofilms in dentin cavities compared with smooth enamel, but effects remained statistically non-significant ($p > 0.05$). Mixed biofilms showed non-significantly decreased pH values compared with SM or LGG biofilms under most conditions (ΔpH = -0.35 ± 0.19 and -0.12 ± 0.19, respectively). For smooth enamel lesions and frequent nutrition supply, mixed biofilms showed a trend of increased pH compared with monospecies biofilms. In general, mixed biofilms yielded higher numbers of colony-forming units per milliliter on MRS than MSB agar (table 1).

Discussion

Besides possible benefits from oral probiotics, their safety is of great clinical relevance. The present study investigated the potential cariogenicity of a common probiotic strain, LGG, and analysed the influence of various factors on demineralization caused by LGG, SM and LGG × SM biofilms. LGG was found to demineralize both enamel and dentin, and LGG × SM biofilms induced similar or even increased demineralization in comparison with SM biofilms alone. Factors like lesion site and avail-

Table 1. Bacterial analysis of biofilms of different species cultured under various conditions (n = 9/group)

Species	Twice daily nutrition supply		Six times daily nutrition supply	
	enamel	dentin	enamel	dentin
SM (BHI)	78±104 (5.1±1.0)	290±559 (4.8±0.4)	1,278±2,468 (4.3±0.1)	1,149±2,583 (3.8±0.1)
LGG (BHI)	98±180 (4.0±0.1)	430±527 (4.0±0.1)	628±288 (4.6±0.1)	1,334±873 (4.5±0.1)
LGG × SM (BHI)	151±296 (4.0±0.4)	553±742 (3.7±0.4)	310±50 (4.7±0.3)	1,364±803 (3.7±0.2)
LGG × SM (MRS)	29±58	567±406	260±60	1,118±308
LGG × SM (MSB)	1±1	239±236	10±10	253±249

Results are expressed as colony-forming units × 10^4 per milliliter (means ± SD) and pH (means ± SD) in parentheses. Bacterial enumeration was performed on BHI, mixed biofilms were additionally analysed using MSB and MRS. Based on ANOVA results, nutrition supply and lesion site were found to significantly influence bacterial numbers: Species: $F = 0.263$, $p = 0.769$; nutrition supply: $F = 8.085$, $p < 0.01$; dental tissue: $F = 5.698$, $p < 0.05$. The pH was not found to be significantly different in different experimental subsets ($p > 0.05$, ANOVA).

ability of fermentable carbohydrates were found to influence demineralization. Thus, we have to reject both our hypotheses.

LGG has been reported to be acidogenic and aciduric [Badet and Thebaud, 2008], with ambiguous reports of its sucrose fermentation capability [Meurman et al., 1995; Hedberg et al., 2008]. The present study confirmed the cariogenic potential of LGG, corroborating with other laboratory [Kaufman et al., 1988] or clinical studies [Kneist et al., 2010]. In vivo, lactobacilli have been found more frequently in pits, fissures or cavitated lesions [Boyar and Bowden, 1985; Bjørndal and Larsen, 2000; Aas et al., 2008], which mechanically retain bacteria and provide a suitable ecological niche, sheltering the bacteria from saliva clearance and maintaining anaerobic and acidic conditions [Bowden and Hamilton, 1989; Badet and Thebaud, 2008; Hedberg et al., 2008]. The present study confirmed such affinity of LGG to dentinal cavities, and found an increased cariogenic potential of LGG in dentin cavities compared with smooth enamel. Furthermore, our analysis of mixed biofilms suggests a partial displacement or suppression of SM by LGG due to the resulting extremely acidic and anaerobic milieu. However, we did not find a significant reduction of total bacterial numbers in such mixed biofilms. Only for smooth enamel lesions and frequent nutrition supply, mixed biofilms showed a trend of decreased bacterial numbers compared with monospecies biofilms. These effects were not reflected by mineral loss comparisons of respective lesions.

The analysis of bacterial numbers is useful to understand how different factors may influence the biofilm itself. Such microbiological analyses have frequently been performed along clinical trials as well, but are of reduced value to assess the cariogenicity of different species under different conditions. Using microradiographic analyses, we found a similar cariogenic potential of SM and LGG monospecies biofilms, but significantly increased cariogenicity of mixed SM × LGG biofilms in dentin cavities. Both species seemed to aggravate each other regarding their demineralization effects, but not their bacterial numbers. Increased acid production has been reported for mixed cultures of SM and *Veillonella* spp. as well as SM and non-probiotic lactobacilli [Noorda et al., 1988; Shu et al., 2000]. In our study, these symbiotic effects were increased in retentive areas and under high nutrition supply frequency. It is highly relevant to find the supply of lactobacilli potentially harmful under cariogenic conditions or in pre-existing cavitated lesions. Such conditions and retentive areas are more likely found in high-risk caries patients, who are a major target population for potential probiotic therapy. The safety of cariogenic species like lactobacilli as oral probiotics can therefore be questioned especially for these patients.

In general, the supply of living lactobacilli for caries prevention is disputable, since they are usually present in great numbers in the oral cavity anyway [Badet and Thebaud, 2008]. Bearing in mind the pathogenesis of caries, a successful suppression or replacement of one cariogenic species might not eliminate caries due to the variability and exchangeability of the species involved in the carious process. The present and other studies indicate that other factors like lesion site or availability of fermentable carbohydrates might affect caries more significantly than the

bacterial composition in the oral cavity [Azevedo et al., 2011]. However, probiotic therapy to prevent or combat oral disease is just evolving, and for example heat-killed bacteria were proven safe, with promising results in animal experiments [Tanzer et al., 2010].

There are several limitations to the present study. First, the complex ecology of oral biofilms cannot be simulated completely in vitro. Observed effects may have been different if other oral species would have been present, since further microorganisms besides SM exert cariogenic effects and may be affected by LGG [Badet and Thebaud, 2008; Takahashi and Nyvad, 2011]. In addition, our analysis of pH of biofilms was not performed directly within the biofilm, but after suspension in medium. Second, a relatively high number of samples was lost during TMR preparation and analyses. Most of these samples fractured during fixation on TMR holders or were lost during the exposure process. It can be argued that such loss may introduce some bias in the obtained results. However, a certain loss of samples had been anticipated during the study design, and the remaining number of samples was found sufficient for statistical analyses. Using alternative assessment methods like transverse wavelength-independent microradiography may allow an analysis of mineral loss without the technical difficulties associated with TMR. Third, mineral loss of enamel samples was relatively small due to the short demineralization period. Longer culturing times might have increased the differences between groups for enamel as well. However, we aimed at having identical protocols for enamel and dentin lesions. Therefore, we decided to not extend the culture time over 10 days, since this would have led to even more pronounced dentinal mineral loss, with associated problems (increased statistical deviation, fragility of samples). Lastly, the daily intake of LGG might have skewed bacterial composition, but was found to be in accordance with clinical protocols, since probiotics are incorporated only short-term and require repeated application [Yli-Knuuttila et al., 2006]. Our analyses showed that is unlikely that the increased cariogenicity of mixed biofilms was caused by a sheer increase in bacterial numbers.

Within the limitations of this study, the probiotic bacterium LGG was found to be cariogenic, with mixed SM × LGG biofilms being significantly more cariogenic than SM monospecies biofilms. The potential cariogenicity of mixed cultures was further increased in dentinal cavities and under frequent nutrition supply. Under these conditions, LGG was not found to reduce caries activity, but rather to contribute to the caries process. The safety of LGG requires further, clinical evaluation.

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Disclosure Statement

The authors declare no conflict of interest.

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